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AUTHOR(S):

Hatano, Hiroyuki

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Oxidative Radiolysis of Amino Acids, Peptides and Proteins in Aqueous Solutions by Gamma Irradiation

Hiroyuki HATANO*

Department of Chemistry, Faculty of Science, Kyoto University

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Radiolytic deamination of amino acids, peptides and proteins in oxygen-containing aqueous solutions by γ -irradiation, was investigated and the reaction mechanism of the oxidative radiolysis was discussed.

Ammonia yield of the deamination of amino acids was not affected over a wide range of irradiation doses while the yield increased together with the concentration of amino acid solutions. α -Amino groups were liberated more easily than β -amino groups which were further more easily than γ -ones, in γ -irradiated amino carboxylic acid solutions. In amino sulfonic acid the deamination of the amino acids took place less readily than in amino carboxylic acid. To the liberation of ammonia in γ -irradiated peptide and protein solutions, not only free amino groups but also peptide bonds were proved to contribute.

α -Keto acid was found to be produced from its parent amino acid by γ -irradiation in oxygen-containing aqueous solutions. α -Keto acid 2,4-dinitrophenylhydrazones were derived from the α -keto acids. They were characterized and determined spectrophotometrically and chromatographically. Reaction yield of α -ketoglutaric acid obtained from γ -irradiated glutamic acid was also affected by irradiation conditions. The yield of decomposed α -alanine was identical stoichiometrically with that of liberated ammonia, while the yield of pyruvic acid from alanine was smaller than that of decomposed alanine and of liberated ammonia. Further decomposition of the pyruvic acid by larger doses of γ -rays, was observed in aqueous solutions.

The mechanism of oxidative deamination of amino acid and peptide, was established and a scheme of radiolysis of proteins was proposed from the results.

INTRODUCTION

It has been found that liberation of ammonia takes place when aqueous solutions of amino acids and proteins are irradiated by ionizing radiations¹⁻⁴⁾. The mechanism of this radiolytic reaction of amino acids and proteins both in oxygen-free and in oxygen-containing solutions, has been studied and various reaction schemes have been proposed⁵⁻¹¹⁾. In the course of radiolysis in oxygen-containing solution, the most important reaction of α -amino acids may be oxidative deamination to give the corresponding α -keto acids from the parent α -amino acids, showing the following reaction:



Among many α -keto acid analogs of α -amino acids, which are rendered difficult to be characterized because of their instability, α -ketoglutaric acid, α -ketoisovaleric

* 波多野 博行

acid and pyruvic acid are possible to be identified as their 2,4-dinitrophenylhydrazone derivatives, for they are relatively less unstable in aqueous solutions¹²⁾. The mechanism of oxidative deamination of the amino acids in aqueous solutions, therefore, may be clarified quantitatively.

Moreover, if the oxidative radiolysis occurs also in oxygen-containing aqueous solutions of peptides, α -keto acids which are derived from the constituent amino acids of the peptide, must be produced in the irradiated peptide solutions. They should also be determined as their 2,4-dinitrophenylhydrazone derivatives, which are separable quantitatively by column chromatography using Hyflo-Super-Cel¹³⁾. Radiolytic cleavage of the peptide bond, thus, can be proved to be brought oxidatively.

In the course of radiolysis of proteins, the oxidative deamination of constituent amino acids of the proteins and the oxidative radiolysis of peptide bonds, may also take place in γ -irradiated protein solutions.

The present paper is concerned with studies on the oxidative deamination of amino acids and the oxidative radiolysis of peptides, and with a discussion on the mechanism of radiolysis of proteins.

EXPERIMENTAL

Materials. Eight amino acids purchased from Azinomoto Co. Inc., Tokyo, were recrystallized from water or a diluted hydrochloric acid solution at least once before use and dried *in vacuo*. Every amino acid and peptide preparation did not show any appreciable amount of contaminative amino acid on its paper-chromatogram. Seven dipeptides, one tripeptide, and other related nitrogenous compounds used in this experiment were obtained from Tokyo Kasei Co. Ltd., Tokyo. Serum albumin of Behring Werke, Berlin, egg albumin and trypsin of E. Merck, Darmstadt, crystalline bacterial amylase of Daiwa Kasei Co. Ltd., Tokyo, and crystalline bacterial proteinase, "Nagarse",* of Nagase and Co. Ltd., Amagasaki, were used in this experiment.

γ -Irradiation. A 50-curie Cobalt-60 source in a Toshiba Teletherapy Unit Model RIT-1** and the Two-kilocurie Cobalt-60 Gamma-Ray Irradiation Facility¹⁴⁾*** were used for γ -irradiation. The dose rates were determined by a Fricke's ferrous-ferri-chemical dosimeter¹⁵⁾ and a physical measurement¹⁶⁾. They were 3.7×10^4 r ± 5 per cent and 1.97×10^5 r ± 5 per cent, per hour, respectively.

The amino acid, peptide and protein solutions from which dissolved air was not expelled, were irradiated with γ -rays in glass tubes (5 cm length and 1 cm dia. or 20 cm length and 2 cm dia.) at room temperature (18–25°C).

Methods. The amount of ammonia in irradiated solutions were determined

* The author wishes to express his thanks to Dr. Hiroshi Saiga, Nagase & Co. Ltd., for supplying "Nagarse" for the present work free of charge.

** The equipment in The University Hospital of Kyoto was used; The author expresses his gratitude to Prof. Tadashi Fukuda, Kyoto University, for offering an opportunity of using it.

*** The equipment in Prof. Shimizu's Laboratory, The Institute for Chemical Research, Kyoto University, was used and the author wishes to express his thanks to Prof. Sakae Shimizu, Kyoto University, for offering use of it.

colorimetrically with an indophenol reagent¹⁷⁾ modified by the author¹⁸⁾ at 625 m μ after collecting quantitatively according to the micro-diffusion method of Conway¹⁹⁾.

Concentration of amino acids in aqueous solutions was measured colorimetrically with a ninhydrin reagent at 570 m μ before and after γ -irradiation by the method of Moore and Stein²⁰⁾ with author's modifications²¹⁾. The amount of amino acid in irradiated solutions was corrected to the amount of ammonia which was colored by the ninhydrin reagent in the course of this determination.

α -Keto acid 2,4-dinitrophenylhydrazones were derived from the keto acids which were produced in irradiated solutions of amino acids and of peptides. They were identified by paperchromatography using a developing solvent, n-butanol: ethanol: 0.1 N sodium carbonate solution containing 0.01 N sodium bicarbonate=1:1:2, v/v²²⁾, and by measuring their ultraviolet absorption spectra of which characteristics were compared with the authentic specimens²³⁾. Quantitative determination of α -keto acids was carried out successfully using Hyflo-Super-Cel column chromatography and spectrophotometric measurements by the method described in the previous papers^{23,24)}.

RESULTS

(1) Ammonia Yield of Deamination from Amino Acids, Peptides and Proteins, in γ -Irradiated Aqueous Solutions

The deaminative reaction, induced by γ -irradiation in oxygen-containing aqueous solutions of amino acids, was a characteristic reaction in which the reaction yield was affected by various irradiating conditions. The ammonia yield obtained from the deamination of glutamic acid, was observed to remain almost constant for a wide range of irradiating doses as shown in Fig. 1. The yield was also found to increase with an increase in the glutamic acid concentration. The result was shown in Fig. 2. Variation of the deamination yields of various amino acids with respect to their chemical configurations in aqueous solutions, was studied obtaining the results presented in Table 1. When oxygen-containing aqueous solutions of peptides,

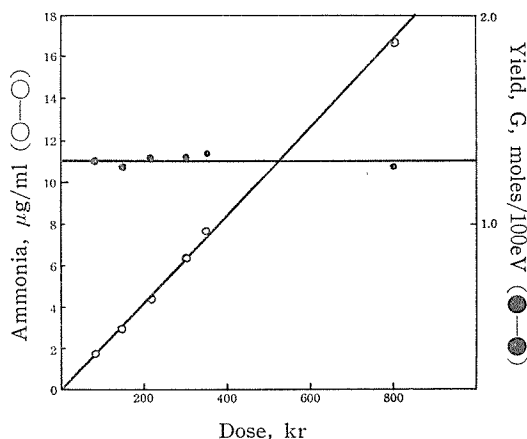


Fig. 1 Ammonia yield from glutamic acid in 31.8 mM solutions after exposing to various doses of γ -rays.

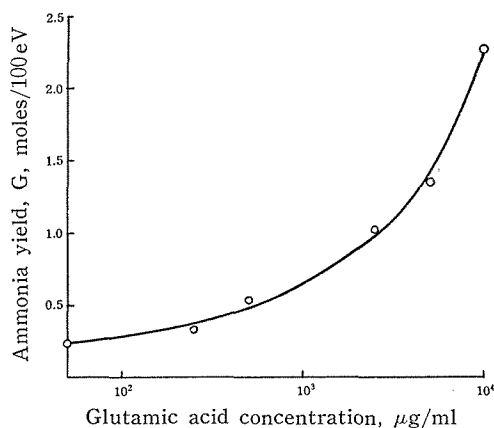


Fig. 2 Ammonia yield from glutamic acid of various concentrations after exposing to 800 kr dose of γ -rays.

Table 1. Ammonia yield from various amino acids in 10 mM solutions after exposing to 800 kr dose of γ -rays.

Amino acid	Chemical configuration	G (NH ₃), moles/100eV
α -Alanine	CH ₃ CH(NH ₂)COOH	2.13
β -Alanine	H ₂ NCH ₂ CH ₂ COOH	1.90
γ -Amino butyric acid	H ₂ NCH ₂ CH ₂ CH ₂ COOH	0.84
Taurine	H ₂ NCH ₂ CH ₂ SO ₃ H	1.41
Leucine free	(CH ₃) ₂ CHCH ₂ CH(NH ₂)COOH	2.33
" sodium salt	(CH ₃) ₂ CHCH ₂ CH(NH ₂)COONa	1.34
" ethyl ester	(CH ₃) ₂ CHCH ₂ CH(NH ₂)COOCH ₂ CH ₃	1.64
" hydrochloride	(CH ₃) ₂ CHCH ₂ CH(NH ₂ HCl)COOH	0.43

Table 2. Ammonia yield from peptides and related nitrogeous compounds in 10 mM solutions by γ -irradiation of 197 kr dose.

	Compound	Chemical structure	G-value moles/100eV
Dipeptide	Glycylglycine	H ₂ NCH ₂ CO-NHCH ₂ COOH	4.02
	Glycyltyrosine	H ₂ NCH ₂ CO-NHCH(C ₆ H ₄ OH)COOH	2.08
	Alanylglycine	H ₂ NCH(CH ₃)CO-NHCH ₂ COOH	1.37
	Alanylalanine	H ₂ NCH(CH ₃)CO-NHCH(CH ₃)COOH	1.30
	Alanylvaline	H ₂ NCH(CH ₃)CO-NHCH[CH(CH ₃) ₂]COOH	0.93
	Alanylleucine	H ₂ NCH(CH ₃)CO-NHCH[CH ₂ CH(CH ₃) ₂]COOH	1.05
Tripeptide	Glutathione	$ \begin{array}{c} \text{H}_2\text{NCHCH}_2\text{CH}_2\text{CO-NHCHCO-NHCH}_2\text{COOH} \\ \begin{array}{cc} & \\ \text{COOH} & \text{CH}_2\text{SH} \end{array} \end{array} $	0.93
Acetyl amino acid	Acetyl glycine	CH ₃ CO-NHCH ₂ COOH	0.73
	Acetylalanine	CH ₃ CO-NHCH(CH ₃)COOH	0.71
	Acetylvaline	CH ₃ CO-NHCH[CH(CH ₃) ₂]COOH	0.73
	Acetylleucine	CH ₃ CO-NHCH[CH ₂ CH(CH ₃) ₂]COOH	0.84
	Acetylmethionine	CH ₃ CO-NHCH(CH ₂ CH ₂ SC ₆ H ₅)COOH	0.80
Benzoyl amino acid	Hippuric acid	C ₆ H ₅ CO-NHCH ₂ COOH	0.45

Table 3. Ammonia yield from protein preparation in 0.5% protein solutions by γ -irradiation of 197 kr dose.

Protein	G-value, moles/100 eV
Egg albumin	1.06
Serum albumin	1.55
Bacterial amylase	1.38
Bacterial proteinase	1.47
Trypsin	2.05

related compounds and proteins, having one or more -CO-NH- bond in their molecule, were irradiated with γ -rays, it was found that liberation of ammonia also took place. The ammonia yield of various peptides and related nitrogenous compounds was summarized in Table 2, and that of protein preparations was given in Table 3.

(2) α -Keto Acids Produced from their Parent Amino Acids and Peptides in γ -Irradiated Aqueous Solutions

α -Keto acid 2,4-dinitrophenylhydrazones were derived from keto acids which were produced in γ -irradiated solutions of the parent amino acid. They had distinguishable characteristics of ultraviolet absorption spectra and showed characteristic chromatographic behaviours. Characterization of pyruvic acid, α -ketoisovaleric acid and α -ketoglutaric acid, which were produced from alanine, valine and glutamic acid respectively in γ -irradiated solutions, was carried out successfully in the previous paper²³⁾. Spectra of 2,4-dinitrophenylhydrazones of the α -keto acid proposed to be produced in several γ -irradiated amino acid solutions, were shown in Fig. 3.

It was shown that when an oxygen-containing aqueous solution of alanylvaline was irradiated with γ -rays, pyruvic acid and α -ketoisovaleric acid, which were the corresponding α -keto acids to the constituent amino acids, alanine and valine, of the peptide, were produced in the irradiated solution. Their 2,4-dinitrophenylhydrazones were derived from them, were separated from each other on a paperchromatogram. They were identified with the authentic specimen as well as in the case of individual amino acids.

The 2,4-dinitrophenylhydrazones of pyruvic acid and α -ketoisovaleric acid, could be separated quantitatively by Hyflo-Super-Cel column chromatography as described in the previous paper²⁴⁾. Column chromatographic separation of 2,4-dinitrophenylhydrazones of pyruvic acid and α -ketoisovaleric acid, which were produced in γ -irradiated alanylvaline solutions after exposing to various doses of γ -rays, was shown in Fig. 4.

When the doses of γ -rays were relatively larger, several peaks of unknown compounds were appeared besides those of pyruvic acid and α -ketoisovaleric acid 2,4-dinitrophenylhydrazones. Two isomers of cis- and trans-pyruvic acid 2,4-dinitrophenylhydrazone, were separated sufficiently to be determined quantitatively on the column.

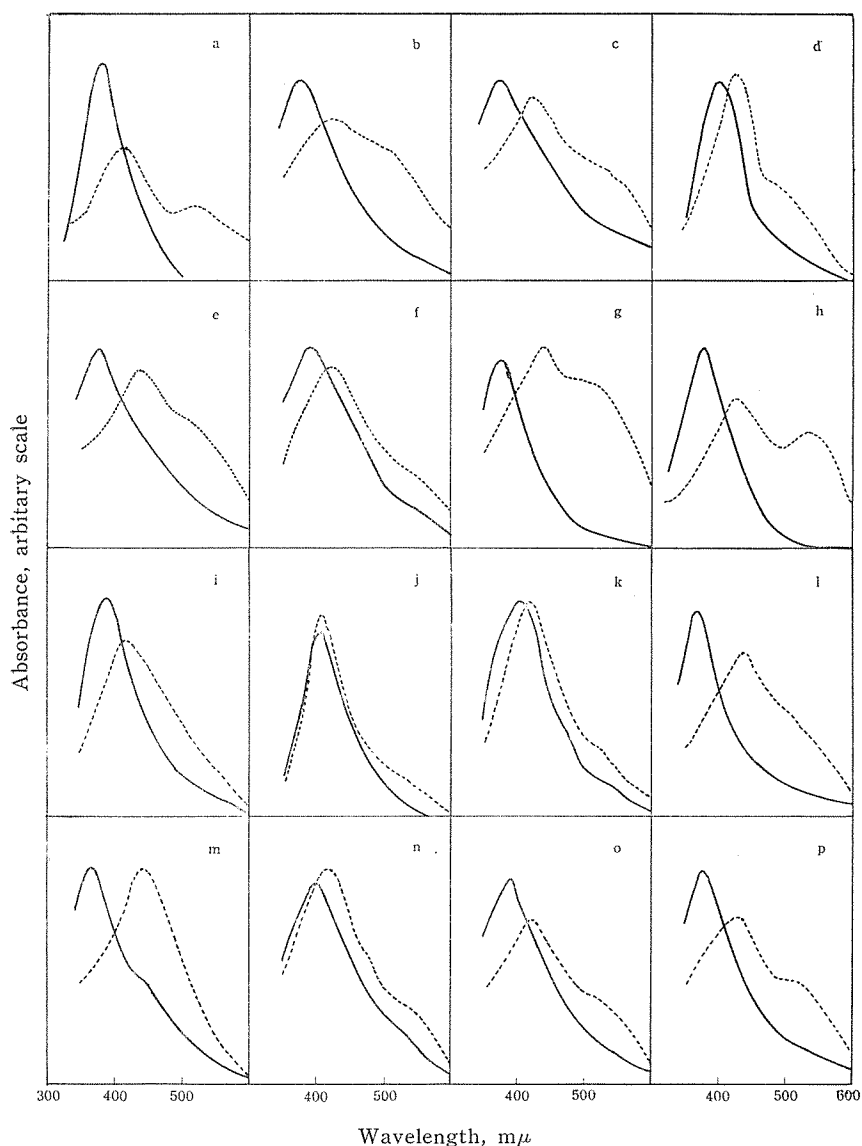


Fig. 3 Absorption spectra of α -keto acid 2,4-dinitrophenylhydrazones derived from α -keto acids proposed to be produced in γ -irradiated amino acid solutions after exposing to 800 kr doses of γ -rays.

Proposed α -keto acid analogs produced from γ -irradiated parent amino acids: a, α -ketoglutaric acid from glutamic acid; b, phenylpyruvic acid from phenylalanine; c, α -keto- ε -aminocaproic acid from lysine; d, β -mercaptopyruvic acid from cysteine; e, β -indolpyruvic acid from tryptophane; f, α -keto- δ -guanidinovaleic acid from arginine; g, oxalacetic acid from aspartic acid; h, α -ketoisovaleric acid from valine; i, β -imidazolylpyruvic acid from histidine; j, β -ketoisocaproic acid from leucine; k, α -keto- δ -aminovaleric acid from proline; l, α -keto- β -hydroxybutyric acid from threonine; m, glyoxalic acid from glycine; n, *p*-hydroxyphenylpyruvic acid from tyrosine; o, β -hydroxypyruvic acid from serine; p, α -keto- γ -methiolbutyric acid from methionine

— presents the spectra of the derivatives in 0.5 *N* sodium carbonate solution;

-----, in a mixed solution of 0.5 *N* sodium carbonate and 0.5 *N* sodium hydroxide.

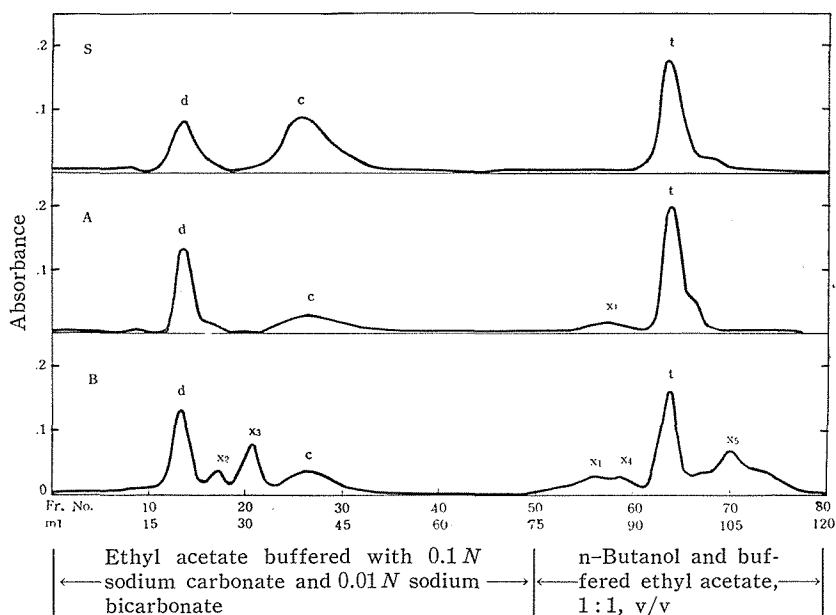


Fig. 4 Hyflo-Super-Cel column chromatographic separation of 2, 4-dinitrophenylhydrazones of pyruvic acid and α -ketoisovaleric acid produced in the γ -irradiated alanylvaline solutions and of the authentic mixture.

The 10 mM alanylvaline solutions were irradiated with γ -ray dose of; A, 1.97×10^5 r. B, 4.5×10^6 r. S, synthetic mixture of the authentic specimens. Details about the chromatographic procedure were presented in the previous paper²⁴. Peak d: derivative of α -ketoisovaleric acid; c: cis-isomer of pyruvic acid derivative; t: trans-isomer of pyruvic acid derivative; and x_1 - x_5 : derivatives of unknown compounds produced by irradiation.

(3) α -Keto Acid Yield from Amino Acids and Peptides in γ -Irradiated Aqueous Solutions

The reaction yield of α -ketoglutaric acid, obtained from glutamic acid in γ -irradiated solutions, was varied with the γ -ray doses exponentially as shown in Fig. 5. The amount of pyruvic acid obtained from alanine in γ -irradiated solutions increased together with the doses of γ -rays till about 4×10^4 r dose under these experimental conditions, but they decreased suddenly in the doses of more than the dose of γ -rays, owing to further decomposition of the keto acid. The result was shown in Fig. 6. The products of the decomposition in the solution irradiated with relatively larger doses of γ -rays, were likely appeared in Fig. 4 presented above.

The amounts of decomposed α -alanine, liberated ammonia and produced pyruvic acid in γ -irradiated aqueous solutions, were determined in various doses of γ -irradiation as shown in Fig. 7. The yield of decomposed α -alanine was identically stoichiometrically with that of ammonia liberated from the parent α -alanine, but not with that of produced pyruvic acid in the deamination process. The yield of pyruvic acid was proved to be about one-third of the yields of α -alanine and of ammonia. Here was also observed the further decomposition of pyruvic acid in

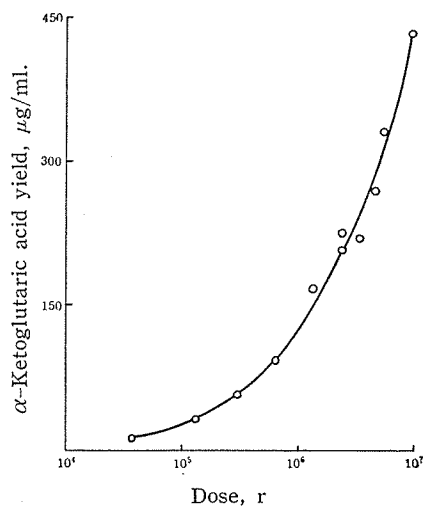


Fig. 5 α -Ketoglutaric acid yield from γ -irradiation of 0.1 *M* glutamic acid solution.

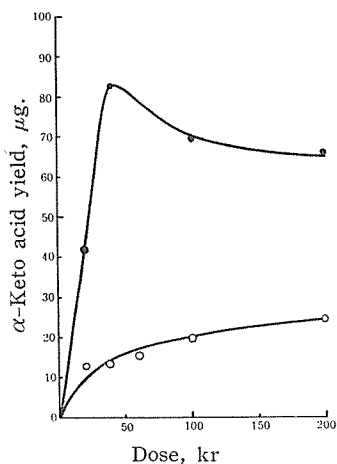


Fig. 6 Pyruvic acid yield (●—●) from parent alanine and α -ketoglutaric acid yield (○—○) from glutamic acid in 10 mM aqueous solutions after exposing to various doses of γ -rays.

γ -irradiated aqueous solutions.

Reaction yield of α -ketoisovaleric acid and pyruvic acid produced from parent alanylvaline in γ -irradiated solutions, was determined colorimetrically after quantitative separation of 2,4-dinitrophenylhydrazones by the column chromatography described in the previous report²⁴, the results being shown in Table 4.

DISCUSSION

As already established, there were many remarkable differences between both radiolytic products of the compounds in oxygen-free aqueous solutions and those

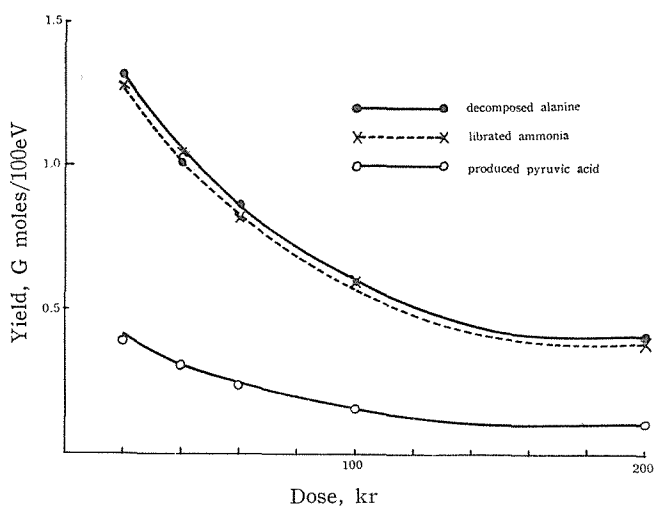


Fig. 7 The amounts of decomposed α -alanine, liberated ammonia and produced pyruvic acid in a 10 mM aqueous solution after exposing to various doses of γ -rays.

Table 4. Reaction yield of keto acid formation from peptide in the 10 mM alanylvaline solution by γ -irradiation.

Dose kr.	Amount of α -ketoisovaleric acid $\mu\text{g.}$	G (K.V.A.) moles/100 eV	Amount of pyruvic acid $\mu\text{g.}$	G (P.A.) moles/100 eV
197	8.67	0.39	11.87	0.71
394	6.09	0.14	16.20	0.48
788	6.36	0.07	17.46	0.26
1379	3.96	0.03	19.62	0.17

in oxygen-containing solutions¹⁰. When the dissolved air was not expelled from the aqueous solutions of amino acids, the deamination reaction of the amino acids by irradiation took place oxidatively in the presence of oxygen²³. All results were obtained under these environmental conditions throughout these experiments.

The constant ammonia yield over the wide range of irradiating doses of γ -rays, suggested that the radiolytic deamination was induced indirectly by the reactive products of irradiated water such as hydroxyl (OH) and hydroperoxyl (O_2H) radicals. The increased yield with an increase in the amino acid concentration, however, showed that the radiation-induced deamination of dissolved amino acids, was caused directly in part on the amino acids together with the indirect action. This result was identical with that described by Dale⁵.

The fact that α -amino acid appeared to be more radiosensitive to deamination than β -amino acid as shown in Dale's experiment⁵, was also confirmed in this experiment. Furthermore, ω -amino acid generally appeared to be deaminated less easily than α -amino acid, and amino sulfonic acid was likely more radio-resistant to the deamination than ordinary α -amino carboxylic acid, by γ -irradiation, judging from the results shown in Table I in which α -, β -, γ -amino carboxylic acid and

even β -amino sulfonic acid, were examined.

A less striking effect on the radiolytic deamination was observed when the amino acid was in the salt and ester forms, and especially in the hydrochloride form in which the dissociation of the amino groups was suppressed. These chemical states of the compounds in an aqueous solution, affected apparently the strength of the C-N bond of amino acid to the radiolytic deamination.

In the case of peptides and related compounds, a large yield of glycylglycine, was observed in accordance with the Dale's observation⁵⁹. Furthermore, glycyltyrosine, which was expected to be more radio-resistant because of its aromatic ring structure, showed a larger yield of ammonia than other alanylpeptides. Generally, glycyl peptides appeared to be more sensitive to radiolytic deamination.

Apparently the C-N bond in acetyl and benzoyl amino acids was observed to be more radio-resistant than the free amino group in amino acids and peptides. The nitrogen of C-N bond in acetyl amino acid, however, contributed more or less to the production of ammonia. Especially the relative small yield of deamination found in hippuric acid, was likely due to the radio-resistancy of aromatic ring structure.

Examined sulfur-containing compounds, glutathione and acetylmethionine, did not show any characteristic behaviours to the radiolytic deamination, though sulfur-containing amino acid and peptide were generally more sensitive to radiation effects²⁵. The small ammonia yield of glutathione, would show somewhat that peptide linkage of the tripeptide did not contribute so much to the liberation of ammonia.

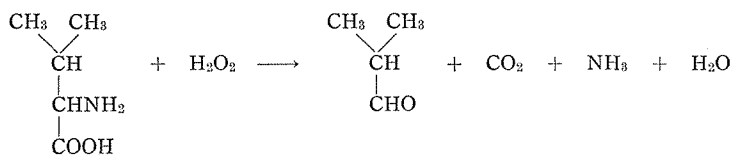
On the protein preparations, radiolytic deamination was observed to occur though the ammonia yield was not so large as expected. The occurrence of deamination would be of importance radiobiologically because of some toxic effects of ammonia on living cells.

Many absorption spectra of 2,4-dinitrophenylhydrazones of α -keto acid analogs, which were produced from the parent amino acid in the γ -irradiated solutions, were given in Fig. 3. The α -keto acids, however, were difficult to be characterized except for α -ketoisovaleric acid and α -ketoglutaric acid because of their instability and similarity of the spectra.

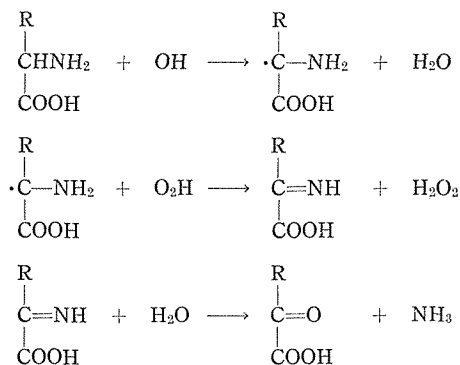
Quantitative determination of α -keto acids in the mixture had been found difficult before they could be separated successfully by Hyflo-Super-Cel column chromatography. This column chromatographic procedure would be applicable to the separation of many other α -keto acids, though many other carbonyl compounds formed in γ -irradiated protein solutions appeared to be too complex to be identified individually.

From the fact that the ammonia yield of deamination of amino acid, was identical with the G-value of degradation of the amino acid, it was shown that the main reaction of the amino acid was the deamination in the course of radiolysis in aqueous solutions.

It had been established that leucine was decarboxylated deaminatively by the action of hydrogen peroxide²⁶ and also of a Fenton's reagent²⁷ to give isovaleryl aldehyde, carbon dioxide and ammonia as follows:

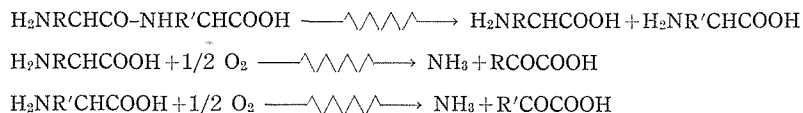


The radiolytic deamination, therefore, would be induced in oxidatively not by the action of hydrogen peroxide and of hydroxyl radicals, but rather by that of hydroperoxyl radicals which were possible to be produced from irradiated air-containing water. The proposed mechanism in which the amino acid was converted to the corresponding keto acid through a hypothetical intermediate, the corresponding imino acid which was possible to be decomposed immediately to the corresponding keto acid, was identical principally with the mechanism of enzymatic oxidative deamination of amino acid as follows:

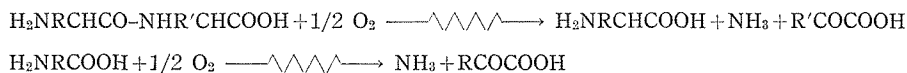


Pyruvic acid yield, however, was found to be about one-third of total deamination yield from alanine. α -Keto acids appeared to be degraded as soon as they were produced in γ -irradiated alanine solutions as shown in Fig. 6. Further degradation of the keto acids and other possible processes of the radiolysis of amino acids in various conditions, were found to be obscure qualitatively and quantitatively.

Formation of carbonyl compounds, especially keto acid corresponding to the constituent amino acid from peptide in the course of radiolysis, was of interest from the following two points. First, the keto acid could be produced oxidatively from the corresponding amino acid, which had been already liberated from the parent peptide in the course of hydrolytic radiolysis, as shown in the previous paper²³⁾, as follows:

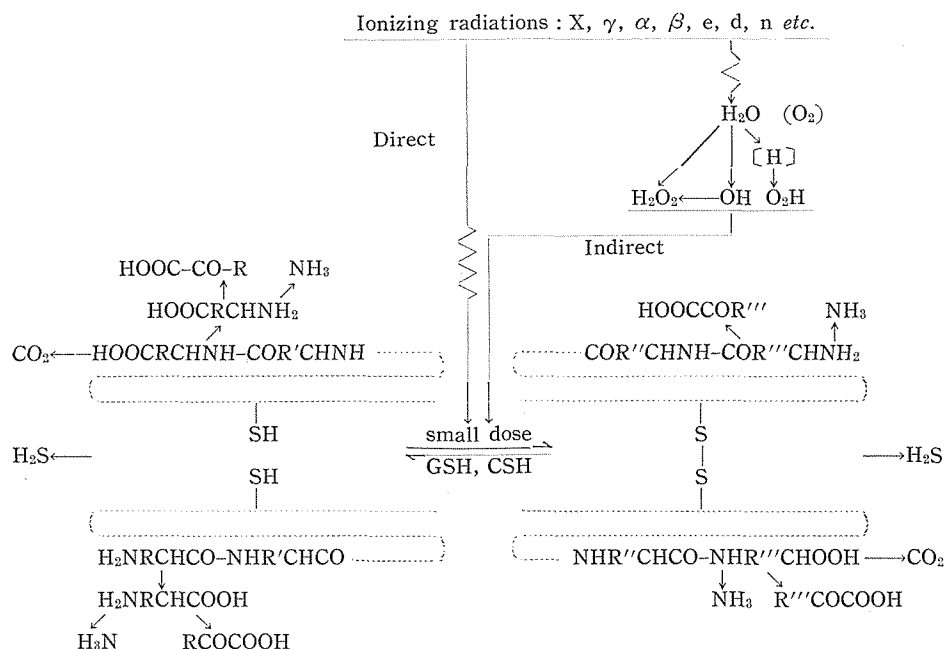


Second, the keto acid would be produced directly from the parent peptide in the course of oxidative radiolysis following further oxidative deamination of fragmentary amino acid:



There should be either of the above two processes or both of them in oxygen-containing aqueous solutions.

From the results obtained in these experiments and in the previous works^{4,11, 23-25, 28-30}, a radiolytic mechanism of protein was proposed conclusively as given in the following scheme:



The radiolysis of peptides and proteins, was of chemical and biological interest because of a close relationship to the radiolytic inactivation and denaturation of biologically active and constituent proteins of living cells by ionizing radiations.

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